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Analysis of *PIG-A* Gene in a Patient Who Developed Reciprocal Translocation of Chromosome 12 and Paroxysmal Nocturnal Hemoglobinuria During Follow-Up of Aplastic Anemia

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The relationships between paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia (AA), and myelodysplastic syndrome (MDS) are not clear. Here we describe a patient, J20, who developed a reciprocal translocation of chromosome 12 and PNH during follow-up of AA. All metaphases in CD59-deficient bone marrow mononuclear cells had the translocation, whereas none of the CD59-sufficient cells had it, indicating that the PNH clone coincided with a cell population bearing the chromosomal aberration. We found a somatic single-base deletion mutation in the *PIG-A* gene of this patient's peripheral blood cells. This is the first patient with PNH with a PNH clone containing a chromosomal translocation. © 1996 Wiley-Liss, Inc.

Key words: myelodysplastic syndrome, glycosylphosphatidylinositol-anchored proteins, decay accelerating factor, CD59

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia (AA), and myelodysplastic syndrome (MDS) are hematopoietic stem cell disorders, and occasionally they complicate each other [1,2]. However, the relationships between these disorders are not completely clear. PNH has been characterized as an acquired hemolytic anemia in which subpopulations of blood cells are deficient in glycosylphosphatidylinositol (GPI)-anchored proteins such as decay accelerating factor (DAF) [3,4] and CD59 [5,6]. The X-linked gene termed *PIG-A* [7,8], which participates in the first step of GPI-anchor biosynthesis [9,10], is mutated in all patients with PNH examined to date [8,11–16]. In some patients with PNH, pancytopenia or bicytopenia is observed, and PNH arises as a common late complication in patients with AA. The prognosis of AA has been improved by immunosuppressive therapy, such as with anti-lymphocyte globulin (ALG). Long-term follow-up of patients with AA, however, has revealed a high incidence of late complications with PNH and MDS [17–20]. Here we describe a patient, J20, who developed PNH and a reciprocal translocation of chromosome 12 during follow-up of AA. We demonstrated a somatic mutation of the *PIG-A* gene in this patient.

CASE REPORT

A 34-year-old-woman presented at Osaka University Hospital for a differential diagnosis of MDS in December, 1993. Pancytopenia was observed in February 1993 in Kyoto Second Red Cross Hospital, and she was treated with anabolic steroids due to a diagnosis of AA (Hb 10.9 g/dl, WBC $2.8 \times 10^3/\mu l$, platelet count $4.4 \times 10^4/\mu l$, and no chromosomal abnormality) from

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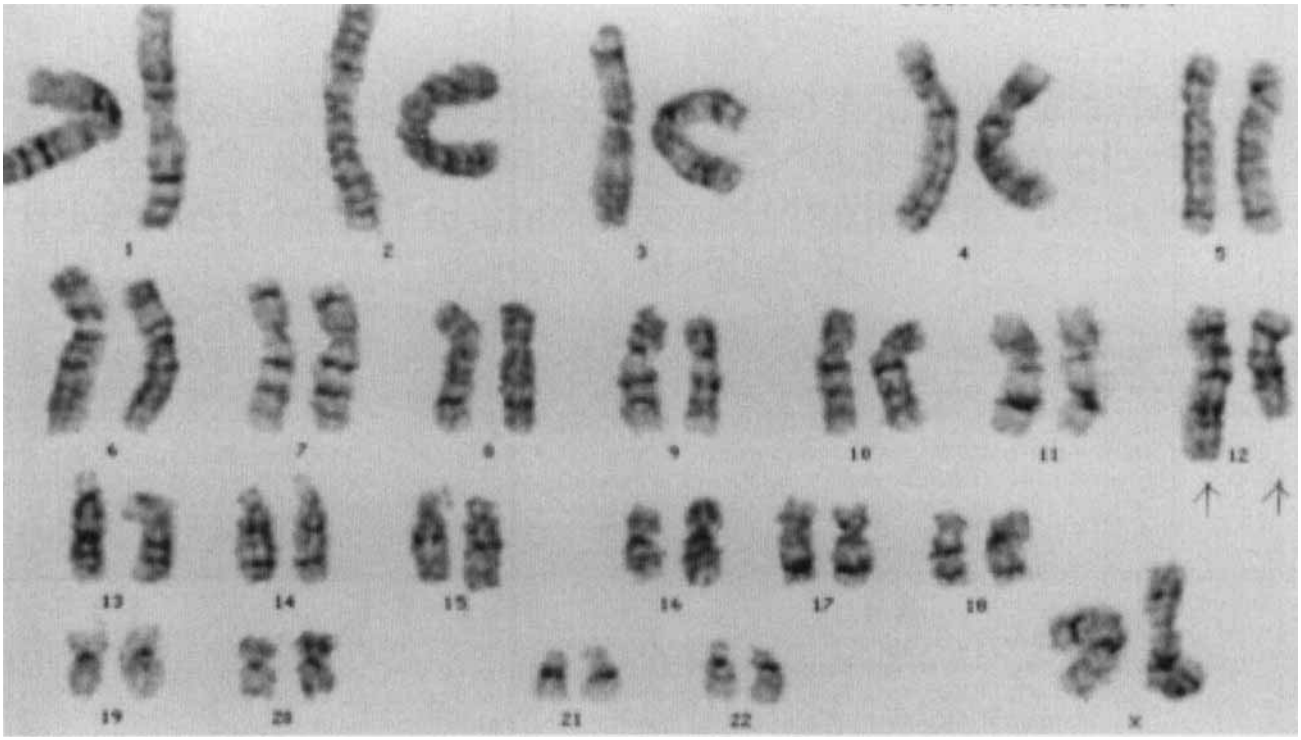

Fig. 1. Reciprocal translocation of chromosome 12, t(12;12)(q13;q15), in blood cells from patient J20.

June 1993. The pancytopenia improved, but a chromosomal abnormality, 46XX,t(12;12)(q13;q15), was detected. Therefore, the transformation to MDS was suspected and she was admitted to the Osaka University Hospital for a closer examination in December 1993. She was hospitalized for an examination in February 1994. There were no particular findings on physical examination. She did not present pancytopenia at that admission. Her Hb was 12.5 g/dl, WBC was $5.1 \times 10^3/\mu l$ with normal differential, and platelet count was $13.5 \times 10^4/\mu l$. Neutrophil alkaline phosphatase was positive in 80% of cells, and the score was 298. The bone marrow was normocellular with a few polynuclear erythroblasts, indicating dysplasia. Other relevant results of laboratory examinations were: lactate dehydrogenase (LDH) 605 U/l (isozyme type I, 33.5%), serum iron (Fe) 89 μg/dl, and total iron binding capacity (TIBC) 472 μg/dl. The level of haptoglobin was below the sensitivity of detection. Direct and indirect Coombs' tests were both negative. We confirmed a translocation of chromosome 12, 46XX,t(12;12)(q13;q15) (Fig. 1), and the fraction of abnormal cells increased from 14.3% (3/21) to 50% (10/20). Erythrocyte life span was slightly reduced (t1/2 = 22.9 days). The ferrokinetic profile did not show signs of AA or MDS, namely ineffective hematopoiesis. The plasma iron half-disappearance time (PIDT 1/2) was 33.7 min, which was slightly shorter than normal (normal range, 60–120 min), but red cell iron utilization ratio (%RCU) was 89.6%, which was within normal range (normal range, 80–100%). Since elevated LDH and low haptoglobin levels indicated intravascular hemolysis, we suspected the development of PNH and analyzed the surface expression of the GPI-anchored proteins, CD59 and DAF, on the patient's blood cells. We found CD59- and DAF-deficient blood cell populations in erythrocytes and granulocytes; 59.7% and 23.4%, respectively, being CD59-negative, and 55.7% and 24.2%, respectively, being DAF-negative (Fig. 2). At the same time, Ham and sucrose hemolysis tests became strongly positive. There was no evidence of hemoglobinuria or of hemolytic episodes. Six months later, these symptoms became evident.

We analyzed the *PIG-A* gene in DNA from her granulocytes [12,21]. Heteroduplex analysis with MDE (mutation detection enhancement) gel (Hydrolink; AT Biochem, Malvern, PA) electrophoresis demonstrated an abnormal profile in a region containing exon 2 (Fig. 3): mobilities of mutant and wild-type clones were slightly different (Fig. 3, lanes 2 and 3), and their mixture showed doublet (Fig. 3, lane 1). Nucleotide sequencing revealed a deletion of one base (guanine) at the end of exon 2 (Fig. 4). This mutation would cause frameshift and a premature stop codon 8nt downstream. However, since the mutation was a base deletion at the 3′ end of exon 2, it might also have

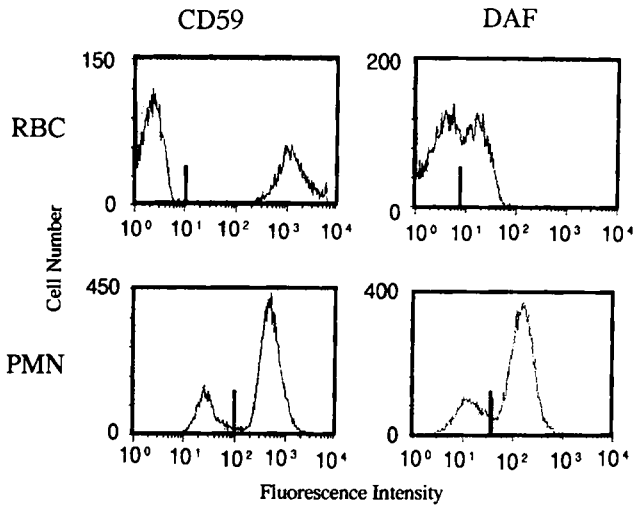

Fig. 2. Deficient surface expression of GPI-anchored proteins on peripheral blood cells from patient J20. RBC (above) and PMN (below) were stained for CD59 (left) and DAF (right).

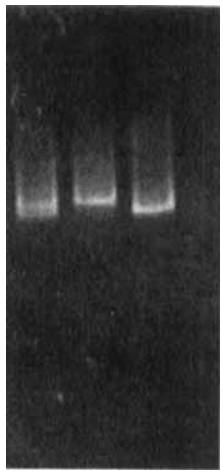

Fig. 3. Heteroduplex analysis using MDE gel. Various regions of the *PIG-A* gene were amplified by PCR from the patient's neutrophils and resolved by electrophoresis in an MDE gel. Results from the 3′ portion of exon 2 are shown. Lane 1, mixture of mutant and wild-type clones; lane 2, mutant clone alone; lane 3, wild-type clone alone.

affected splicing, presumably resulting in a decrease of the major transcript.

At the diagnosis of AA, the patient's karyotype was normal, and Ham and sucrose hemolysis tests were negative. Later, the chromosomal abnormality and cells with the PNH phenotype appeared. To understand the relationship between the PNH clone and cells bearing the chromosomal translocation, we analyzed the karyotype of CD59-

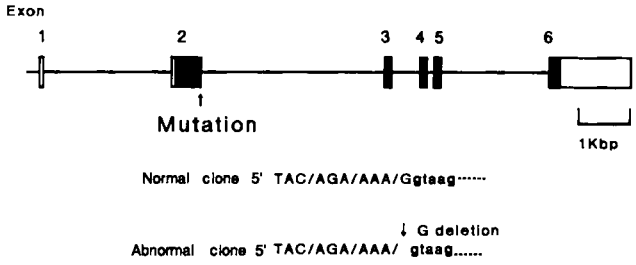

Fig. 4. Somatic mutation of the *PIG-A* gene in exon 2, found in peripheral blood cells from patient J20. Schematic representation of the *PIG-A* gene is shown. Below are normal and mutant nucleotide sequences of a region of exon 2 that contains a mutation (arrows), a part of exon 3, and an intron in between. Exon and intron sequences are in upper- and lowercase letters, respectively. In scheme of *PIG-A*, boxes represent exons. Solid and open areas indicate coding and untranslated regions, respectively.

deficient and -sufficient cells. Samples were obtained by bone marrow aspiration from the patient with informed consent. Mononuclear cells were separated from the bone marrow cells and stained for CD59. CD59-negative and -positive populations were separated by fluorescent cytometry using a FACStar (Becton-Dickinson, Lincoln Park, NJ), and the karyotype was analyzed. All metaphases in the CD59-deficient population had the chromosomal abnormality, whereas none in the CD59-positive population had it. The PNH clone, therefore, coincided with a cell population bearing a translocation of chromosome 12.

DISCUSSION

We demonstrated a somatic mutation of the *PIG-A* gene in a patient who developed PNH and a translocation of chromosome 12 during follow-up of AA. Initially, we suspected MDS from the chromosomal aberration, but this was ruled out by results of the ferrokinetic study. Recent studies have shown that patients with AA who have been treated with immunosuppressive therapy have a high incidence of late complications with PNH and/or MDS [17–20]. Since elevated LDH and low haptoglobin levels indicated intravascular hemolysis, we suspected the development of PNH and demonstrated the deficient surface expression of GPI-anchored proteins on blood cells from this patient. At the same time, acid and sucrose hemolysis tests became strongly positive, which are diagnostic signs of PNH. PNH is an acquired hematopoietic disorder characterized by a deficiency of GPI-anchored proteins on hematopoietic cells. We cloned an X-linked gene termed *PIG-A* [7,8], which participates in an early step of GPI-anchor biosynthesis [9,10]. To date, more than 50 patients with PNH have been analyzed for the

PIG-A gene, and its somatic mutations have been detected in all patients [8,11–16].

The most likely reason for this uniformity is that among the genes involved in GPI-anchor biosynthesis, only the *PIG-A* gene is localized on the X chromosome [8], whereas all other genes are autosomal [22]. Therefore, one hit of a loss-of-function mutation in *PIG-A* could cause a GPI-anchor deficiency (because even in females, one of the X chromosomes is inactivated), whereas two-hit mutations are necessary for the autosomal genes. In fact, three other genes have been cloned and proven to be autosomal.

The patient reported here had a dominant clonal population, with the GPI-anchor deficiency and the reciprocal chromosome 12 translocation. We initially suspected that one GPI-anchor biosynthesis gene might be located on chromosome 12 and that it was disrupted somatically by the translocation, although no genes relevant to GPI-anchor biosynthesis have been localized on chromosome 12. We therefore checked whether the *PIG-A* gene was abnormal. Like other patients with PNH, this patient also had a somatic mutation in the *PIG-A* gene (Fig. 4). Because only one mutation in *PIG-A* would result in a GPI-anchor-deficient phenotype, these data indicated that an abnormality of chromosome 12 may not be responsible for the deficiency of the GPI anchor.

In patients with PNH, GPI-anchor-deficient cells occupy a significant fraction of all lineages of peripheral blood cells, indicating that hematopoietic stem cells are affected.

Analysis of the $CD34^+$ $CD38^-$ bone marrow cell population which contains hematopoietic stem cells has indicated that GPI-anchor-deficient cells are dominant at this stage. The abnormal clone(s), therefore, must dominate the normal clones at the stem cell level [23].

Although the *PIG-A* gene is responsible for the GPI-anchor deficiency in PNH [8,11], whether or not the *PIG-A* gene is also involved in clonal dominance is unknown. Bessler et al. [14,16] have reported two examples of two independent *PIG-A* mutants in a patient with PNH. We also found a patient with PNH bearing two independent clones [12]. These facts suggest that *PIG-A* mutants can always become dominant and, therefore, that *PIG-A* mutations are somehow related to clonal dominance. There are three explanations for the dominance of the abnormal clone bearing the *PIG-A* mutation. First, GPI-anchor-deficient cells bearing *PIG-A* mutations have an intrinsic ability to expand. The percentage of GPI-anchor-deficient cells in the periphery varies among patients with *PIG-A* mutations, causing a complete loss-of-function and they are relatively stable for a long time in each patient. Thus, the dominance of GPI-anchor-deficient cells is not determined by the function of *PIG-A* protein itself, suggesting that the *PIG-A* mutation alone is not responsible for clonal expansion. The second explanation is that in addition to the *PIG-A* mutation, abnormal clones have another abnormality (or abnormalities) that is (or are) responsible for expansion. If there are several other abnormalities, patients with complete loss-of-function of *PIG-A* would have different percentages of GPI-anchor-deficient cells. The third explanation is the positive selection of GPI-anchor-deficient cells. If some condition(s) of the bone marrow suppresses normal clones more severely than abnormal clones, the relative dominance of the former would be established.

In patient J20, all GPI-anchor-deficient cells had the chromosome 12 translocation. If the translocation conferred a growth advantage of the GPI-anchor-deficient cells, the second explanation would be supported. However, we cannot exclude the possibility that the translocation is totally irrelevant to the pathogenesis of PNH.

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